Immune Modulation by cross-linked Bovine Colostrum in vitro and in vivo

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Abstract

Bovine colostrum (BC) is the early thick yellow fluid produced by cows during the first several days after birth of the calf. BC is different from "mature" milk as it contains nutrients and immune factors, which strengthen the immune system of the newborn calf in its first week of life. BC has a long history of use in traditional medicine throughout the world and is currently also used in a topical cream for the treatment of immuno-related skin problems such as atopic dermatitis or psoriasis. However, despite a large amount of literature concerning the properties of human or bovine colostrum, there are only few reports on the effects of colostrum on the human immune system.

In this study, the effects of cross-linked BC containing hyaluronic acid on the T-cell/macrophage interplay were investigated in human peripheral blood mononuclear cells (PBMC) and the results were compared with the effects of euxyl 9010, which was used to preserve the BC ingredients. The cross-linked BC preparations showed significant immunomodulatory effects on unstimulated and phytohaemagglutinin (PHA)-stimulated human PBMC by modulating tryptophan degradation and formation of neopterin in a biphasic manner. These results could be relevant for some of the beneficial effects of BC observed in the treatment of skin lesions of patients with atopic dermatitis or psoriasis. However, additional studies are needed to confirm the first positive results obtained in patients.

Key words: immune modulation, Bovine Colostrum, IFN-γ, neopterin

Introduction

Colostrum is the premilk fluid produced by mammals at birth. Colostrum contains the ideal composition of immunoglobulins IgA, IgM and IgG, growth factors, antibodies, vitamins, minerals, enzymes and amino acids to protect the newborn from viral and bacterial infections and to promote immune system function at birth (1-3). Bovine colostrum (BC) contains also growth factors such as insulin-like growth factor (IGF) I and II, transforming growth factor-β (TGF-β) or epidermal growth factor (EGF), and pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (4-7). Additionally, BC also contains anti-inflammatory factors such as IL-1ra, sIL-1RII and sCD14, and high amounts of antimicrobial lactoferrin (1.5-5 g/L) (8-13).

The documentation of effects of BC on the human immune system is sparse. After weak antigenic stimulation of human peripheral blood mononuclear cells (PBMC), natural BC was shown to induce IL-12 and to enhance IFN-γ production (14). Furthermore, a commercially available BC protein concentrate was shown to stimulate secretion of IFN-γ, interleukin-10 (IL-10), and interleukin-2 (IL-2) in PBMC cultures, thereby promoting a Th1-type immune response. In contrast, pretreatment of PHA-stimulated PBMC with BC, enhanced the secretion of IL-10 and IL-12 and suppressed production of IFN-γ and TNF-α (15).

Lactoferrin, of which high amounts are present in BC, acts as an immune mediator regulating target cell responses, including those involved in oxidative stress and systemic inflammatory responses. Lactoferrin can induce mediators of the innate immune response, thereby influencing adaptive immune cell function. In
this regard it was shown that daily oral administration of lactoferrin can support the immune system response through antioxidant mechanisms (16). This could be important in allergic disorders, such as asthma, rhinitis, and atopic dermatitis, in which oxidative stress plays a major role (17). Preclinical and clinical studies have demonstrated that lactoferrin can inhibit dermal inflammatory cytokine production, indicating that lactoferrin may represent a potent anti-inflammatory protein at local sites of inflammation (11). Thus, it was assumed that using bovine colostrum ingredients, which contain high amounts of lactoferrin in a topical cream, might also help in reinstating the immunological balance of the skin of patients with immune-related diseases such as psoriasis or atopic dermatitis (AD).

Although the immunohistological characteristics of the skin in the acute phase of AD correspond to a Th2-type immune response pattern, chronic lesions are associated with Th1-type immunity, as it is the case in psoriasis (18). As a consequence, both diseases show good responsiveness to T-cell directed treatment regimens (19-22).

During Th1-type immune responses, large amounts of cytokines such as IL-2 or IFN-γ are released by activated T-cells, which mediate pro-inflammatory functions critical for the development of cell-mediated immune responses. Besides other pathways, T-cell derived IFN-γ induces also activation of the enzyme indoleamine 2,3-dioxygenase (IDO) in macrophages, that converts tryptophan into N-formylkynurenine, which subsequently is deformylated to kynurenine (23). IDO plays a central role in the suppression of cytokine production, indicating that this enzyme is a strong suppressive capacity on Th1-type immune response in unstimulated PBMC (27). Recently it was shown that BC containing low or high amounts of lactose have demonstrated both immune stimulatory and inhibitory effects on T-cell responses critical for the development of cell-mediated immunity (28). In diseases such as psoriasis or atopic dermatitis, the concentration of IFN-γ was higher than in healthy and active control groups (28).

A basic cream containing either BC powder, cross-linked BC, or BC cross-linked together with hyaluronic acid (HA), was prepared as a water-oil emulsion. HA is one of the natural polymers belonging to the sulfated glycosaminoglycans class and represents the main component of the dermis extracellular matrix. Besides having important mechanical and structural functions, it plays a key role in the wound healing process, inducing fibroblast proliferation and stimulating collagen metabolism during the granulation phase of the healing process with a subsequent increase in collagenous fibers. The cream was then applied to several volunteers suffering from either atopic dermatitis or psoriasis.

Materials and Methods

Chemicals

Low lactose colostrum powder was obtained from New Zealand marketed by www.neovite.com (England) (28). To preserve the colostrum extract, Euxyl® PE 9010, a mixture of phenoxy ethanol and ethylhexylglycerin (Schülke & Mayr, www.schuelke-mayr.com) (29), was used at a concentration of 3%. The final concentration of Euxyl® PE 9010 in the finished cream was 1%. HA was purchased from Novozymes, Bagsvaerd Denmark and DHA (dihydroxyacetone) was obtained from Sigma-Aldrich.

Preparation of nanoparticles using colostrum powder, Hyaluronic acid (HA) and dihydroxyacetone (DHA):

One gram of low lactose colostrum was dissolved in 90 ml of 10 mM sodium phosphate buffer (pH 7.4). 0.1g HA was dissolved separately in 10 ml of phosphate buffer and then mixed with the colostrum solution. To this solution 0.1 g of DHA was added and stirred at 500 rpm for 1h at ambient temperature to promote crosslinking of proteins. The cross-linked proteins were then centrifuged at 30,000 g for 1h at 4°C (Sorvall centrifuge, SLA 1500 rotor). The pellet was washed 3 times with phosphate buffer and resuspended in phosphate buffer for further analyses. Nanoparticles were identified by atomic force microscopy (AFM). Samples were spotted on mica sheet and left to dry partially for 5 minutes. AFM was performed with a scan frequency of 5 Hz and scan size 5 μm, using the tapping mode system.

Isolation and stimulation of human PBMC

PBMC were isolated from whole blood obtained from healthy donors, of whom informed consent was obtained that their donated blood unit was used for sci-
Scientific purposes if not otherwise used. Separation of blood cells was performed using density centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). After isolation, PBMC were washed three times in phosphate buffered saline containing 0.2% EDTA [0.5 mmol/L]. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), 1% of 200 mmol/L glutamine (Serva, Heidelberg, Germany) and 0.1% of gentamicin (50 mg/ml, Bio-Whittaker, Walkersville, MD) in a humidified atmosphere containing 5% CO₂ for 48h. This procedure was observed earlier to reveal best reproducible results when applied for testing of anti-inflammatory effects of compounds or drugs (27).

Average tryptophan content in the supplemented RPMI 1640 medium was 31.5 μmol/L. For each of the three experiments run in duplicates, PBMC were freshly prepared. Isolated PBMC were plated at a density of 1.5 x 10⁶ cells/ml in supplemented RPMI 1640, preincubated for 30 minutes with or without BC preparations and stimulated or not with 10 μg/ml PHA for 48h (28).

Measurement of tryptophan, kynurenine, and neopterin concentrations

After incubation of cells for 48h, supernatants were harvested by centrifugation and tryptophan and kynurenine concentrations were measured by high performance liquid chromatography (HPLC) using 3-nitro-L-tyrosine as an internal standard (30). To estimate IDO activity, kyn/trp was calculated and expressed as μmol kynurenine/mmol tryptophan (31). Neopterin concentrations were determined by ELISA (BRAHMS, Henningsdorf/Berlin, Germany) according to the manufacturer’s instructions with a detection limit of 2 nmol/L.

Measurement of cell viability

After incubation of PBMC, cell viability was measured by MTT-test (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; Sigma, Vienna, Austria) and by trypan blue exclusion method in three experiments done in triplicates. No toxicity could be observed at the concentration range applied (data not shown).

Statistical analysis

For statistical analysis, the Statistical Package for the Social Sciences (version 14 SPSS, Chicago, Ill, USA) was used. Because not all data sets showed normal distribution, for comparison of grouped data non-parametric Friedman test and Wilcoxon signed ranks test were applied. P-values below 0.05 were considered to indicate significant differences.
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± 22.3%. Lactoferrin induced only a moderate increase of neopterin at 0.2 or 2 mg/ml to 125 ± 2.8% or 132 ± 4.1%, respectively (28).

Nanoparticles of colostrum containing HA cross-linked with DHA strongly induced IDO activity at 2 mg/ml to 1122.0 ± 356%, which was lowered by the addition of the preservative euxyl to 296.6 ± 70.3% (Fig. 2A). Treatment of PBMC with euxyl alone suppressed spontaneous IDO activity to 67.9 ± 6.3% or 42.3 ± 4.75% at doses of 2 and 20 mg/ml, respective-

Figure 1: Atomic force microscopy picture of cross-linked nanoparticles. 3 l of sample was placed on a mica slide, allowed to partially dry and was scanned using the tapping mode system at a scan rate of 0.5 Hz and scan size of 5 µm. The size of the nanoparticles was in the range of 60-180 nm.

Figure 2: Kynurenine to tryptophan ratio (left) and neopterin formation (right) expressed as % of unstimulated control in human peripheral blood mononuclear cells treated or not with increasing concentrations of bovine colostrum nanoparticles containing hyaluronic acid with or without preservative euxyl and euxyl alone for 48h. Results shown are the mean values ± S.E.M. of three independent experiments run in duplicates (*p <0.05).
ly. Neopterin levels also increased strongest after treatment of cells with colostrum containing no euxyl (2mg/ml: 196 ± 21.2%; 20 mg/ml: 209 ± 26.9%), which was reduced by the addition of euxyl to 128 ± 12.7% and 169 ± 21.6% at 2 and 20 mg/ml, respectively. Application of euxyl alone diminished neopterin formation at doses of 2 mg/ml to 90.4 ± 4.1% and at 20 mg/L to 76.1 ± 1.6% (Fig. 2B).

Effect of BC preparations on tryptophan metabolism and neopterin formation
in PHA-stimulated PBMC

Upon treatment of PBMC with 10 µg/ml PHA for 48 h, tryptophan content in the supernatant decreased to 8.2 ± 2.0 µmol/L whereas kynurenine concentrations increased concomitantly to 9.8 ± 1.3 µmol/L, indicating an approximately 50-fold increase of IDO activity (Kyn/trp: 1312 ± 45.9 µmol/mmol) (28). Within the same supernatants, neopterin concentrations raised about 3.6-fold to a level of 13.2 ± 3.6 nmol/L. Pre-treatment of PHA-stimulated PBMC cultures with BC preparations containing low or high amounts of lactose or lactoferrin alone revealed a strong and dose dependent capacity to suppress PHA-induced tryptophan degradation. BC with low amounts of lactose showed the strongest inhibitory effect on IDO enzyme activity (0.2 mg/ml: 25.4 ± 5.3%) followed by the effect of lactoferrin (0.2 mg/ml: 34.0 ± 9.7%) and BC with higher amounts of lactose (46.0 ± 16.6%). At higher concentrations of 2 or 20 mg/ml all preparations almost completely counteracted PHA-stimulated tryptophan degradation in the same rank order of activity (28). Mitogen induced neopterin formation was also diminished by these BC preparations and lactoferrin, although with lower potency as compared to the effects on tryptophan degradation. Again, BC with higher amounts of lactose showed the weakest inhibitory effect on PHA-stimulated neopterin formation, exerting a significant inhibition to 70.5 ± 7.2% only at a dosage of 20 mg/ml. The potency of BC with low amounts of lactose, taking effect at 2mg/ml (64.0 ± 4.8%) and 20 mg/ml (37.1 ± 3.0%), were comparable to the effect of lactoferrin (2mg/ml: 61.3 ± 11.7%; 20mg/ml: 31.1 ± 3.9%) (28).

The BC nanoparticle preparation containing HA, showed a lower capacity to counteract PHA-induced tryptophan degradation and neopterin formation in PBMC. Pre-treatment of cells with BC containing no euxyl suppressed PHA-stimulated IDO activity at 2 mg/ml to 78.2 ± 5.0% and to 28.9 ± 6.4% at 20 mg/ml, which was slightly enhanced by the addition of euxyl to 74.1 ± 11.9 and 17.7 ± 6.1%, respectively. Euxyl alone suppressed PHA-stimulated tryptophan degradation at doses of 2 mg/l to 60.1 ± 11.0% and almost completely at 20 mg/ml to 2.9 ± 0.4% (Fig. 3A).

Interestingly, this BC preparation did not affect PHA-induced neopterin formation. A significant reduction of PHA-stimulated neopterin formation with BC containing euxyl, at a dosage of 20 mg/ml to 73.0 ± 5.7%, may possibly be linked to the suppressing capacity of euxyl (2mg/ml: 85.4 ± 3.5%; 20 mg/ml: 34.2 ± 4.2%; Fig. 3B).

Application of BC cream on volunteers suffering from atopic dermatitis or psoriasis

Forty volunteers with psoriasis and 30 patients with atopic dermatitis were treated over a period of 6 months with a cream containing cross-linked colostrum nanoparticles. After one week of applying the colostrum cream, 90 % of volunteers suffering from psoriasis observed a marked decrease in itching and scaling. In 45 % of the volunteers with psoriasis, there was an improvement or even total clearance of psoriatic lesions within 4-6 months after treatment in some of the patients.

The atopic dermatitis volunteers, who used the cross-linked colostrum cream, suffered from moderate to severe atopic dermatitis. In these patients, the onset of efficacy was observed after 2-4 days. The greatest benefit reported, was the alleviation of itching in more than 95 % of the patients, especially during the night. About 65 % of the patients with atopic dermatitis showed a strong reduction of eczema after one week of treatment. No adverse side effects were observed.

There was a marked improvement of symptoms in most of the volunteers who regularly applied the cream, sometimes atopic dermatitis inflammation disappeared within 4 days. One patient suffering from psoriasis with severe itching for over 40 years has also become symptom-free for four months.

Discussion

Cross-linking BC with HA in combination with DHA increased the amount of protein in the nanoparticle preparations as compared to the use of DHA only. Protein degradation by proteases generally occurs within minutes to several hours. The fact that degradation of the nanoparticle preparation in presence of HA by proteases was delayed until up to 20h of treatment, suggests that HA not only contributes to an increase of cross-linked protein, but also to strengthen the cross-linking process. The in vitro model of activated PBMC has been well established in clinical immunology for several decades and allows standardization of T-cell activation and T-cell/macrophage interactions, which is highly relevant in the pathogenesis of immunological disorders. Immune components of BC comprise, amongst...
others, Th-1- as well as Th-2-type cytokines, which play important roles as mediators in the regulation of immune and inflammatory responses. In general, cytokines do not regulate normal cellular homeostasis, but alter cellular metabolism during times of perturbation, e.g. in response to inflammation. Pro-inflammatory cytokine IFN-γ is probably the most important multiplier of anti-microbial and anti-tumoral host defence, producing a variety of physiological and cellular responses such as degradation of tryptophan and formation of neopterin. Increased tryptophan degradation and neopterin production were found in patients during diseases which are associated with Th1-type immune activation such as infections, autoimmune diseases, malignant disorders, and during allograft rejection episodes (32).

The present study shows a bidirectional capacity of all BC nanoparticle preparations investigated, to either induce or to inhibit a Th-1 type immune response in human PBMC. In unstimulated PBMC, all BC preparations stimulated tryptophan degradation and formation of neopterin, while tryptophan degradation in PHA-stimulated PBMC was found to be strongly suppressed. However, the capacity to counteract PHA-induced neopterin formation was obviously weaker or almost lacking. Higher amounts of lactose present in BC attenuated the stimulatory potential in unstimulated PBMC as well as the inhibitory activity in PHA-stimulated cells. In unstimulated PBMC, lactoferrin suppressed tryptophan degradation only at high doses and induced a slight increase of neopterin secreted into the supernatant. The suppressive activity of lactoferrin in PHA-stimulated PBMC was comparable to the effect of BC containing low amounts of lactose and the presence of the preservative euxyl attenuated the stimulatory capacity of BC but did not affect its suppressive activity on tryptophan degradation in PHA-stimulated cells.

These results correspond well with data of Shing and others, who reported a stimulatory capacity of a BC protein concentrate on IFN-γ secretion in unstimulated human PBMC, whereas co-treatment of PHA-stimulated PBMC with BC, suppressed production of IFN-γ (15). Furthermore, earlier experiments of Biswas and others, revealed also a stimulatory effect of BC on IL-12 production and an enhancing effect on antigen-stimulated IFN-γ formation in PBMC (14). However, no inhibitory effect of BC on antigen-induced IFN-γ production could be detected earlier (14), which may result from the lower concentration range applied (0.1-10 µg/ml), in comparison to the concentrations used by us (0.2-20 mg/ml) or Shing et al. (12.5-50 mg/ml) (15).

A potential role for BC as a therapeutic regimen in cutaneous inflammatory diseases may be assumed because of the high content of lactoferrin (1.5-5 g/L), an 78 kD protein that has been shown to transport essential iron to haematopoietic cells and to prevent harmful viruses and bacteria from getting the iron they need for their growth (9, 11). Receptors for lactoferrin are found on skin, intestinal tissues, monocytes, macrophages, neutrophils, lymphocytes, platelets and...
on some bacteria (9, 11). By binding free iron, lactoferrin may also act as an antioxidant, protecting the immune cells against free radicals produced by themselves in areas of inflammation or infection (33). Moreover, it also binds and neutralizes bacterial lipopolysaccharide, and thus reduces production of cytokines in response to inflammation or infection (11, 34). Lactoferrin was shown to be produced at high levels in patients with allergic reactions of the skin and to inhibit allergen-induced Langerhans cell migration and cutaneous inflammation in humans (35, 36).

Atopic dermatitis (AD) and psoriasis are the two most common chronic inflammatory skin diseases found in the general population (19). However, their mechanisms for skin inflammation and propensity for skin infection are quite different. In AD, the immune response is mainly Th2-type contributing to high IgE production (19), while in psoriasis the immune response usually exhibits more a Th1-type profile (37).

A conspicuous common attribute to the pathophysiology of AD and psoriasis lesions is that they share some immunological aspects such as dermal infiltration of T cells, macrophages and dendritic cells (38-40). Persistent skin inflammation in chronic lesions of AD patients involve repeated scratching and tissue damage, which switches the immune response from a Th-2 to a Th-1 type cytokine pattern, which is also characteristic for psoriatic lesions (18, 19, 41).

Figure 4: Patient with psoriasis before treatment with cream (upper left) and 4 months after treatment with cream (upper right). Another patient with psoriasis before treatment with cream (middle left) and 4 months after treatment with cream (middle right). Patient with atopic dermatitis before treatment with cream (lower left) and 4 days after treatment with cream (lower right)
Consequently, both diseases show good response to T-cell directed treatment regimes (19-22). In such a way, infliximab, an anti-TNF chimeric monoclonal antibody of the IgG1 class, has been shown to be effective in diseases associated with a Th-1 type immune response, such as rheumatoid arthritis, Crohn’s disease and psoriasis, but has also been evaluated in other inflammatory dermatoses, such as severe atopic dermatitis, pityriasis rubra pilaris, pyoderma gangrenosum and cutaneous sarcoidosis (21, 22).

The present study shows that BC exerts a biphasic mode of action by promoting a Th-1 type immune activation in unstimulated PBMC, as well as a strong suppressing capacity on Th-1 induced biochemical pathways in mitogen stimulated human PBMC. The suppressive potential of BC on PHA induced tryptophan degradation may be related to the content of lactoferrin, which was shown to be effective in the same concentration range (28). The addition of eukyl, could be shown to be suitable as a preservative for BC, since the stimulatory activity of BC was reduced but the suppressive activity was not affected, most probably because eukyl itself showed inhibitory activity on PHA-induced tryptophan degradation. Although this effect of BC to suppress Th1-type immune responses has been gathered from in vitro experiments at comparatively high concentrations, we assume that at least when topically applied to the skin lesions of AD or psoriasis patients, all components of BC are present at effectual concentrations to down-regulate the activity of infiltrated T-cells, macrophages and other mononuclear cells.

Still due to absence of any statistical evaluation of the in vivo results, these aspects are still to be regarded as preliminary, and certainly further studies are needed to confirm the findings of the beneficial effects of BC as treatment regimen for AD or psoriasis patients. Nevertheless, the first in vivo observations using the BC preparation provide a promising impression.

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References

15 Shing CM, Peake JM, Suzuki K, Jenkins DG, Coombes JS. Bovine colostrum modulates cytokine production in human peripheral blood mononuclear cells stimulated with lipopolysaccha-